Journal of Medical Virology 50:35–41 (1996)

B-Cell Epitopes in Hypervariable Region 1 of Hepatitis C Virus Obtained From Patients With Chronic Persistent Hepatitis

Yasunari Nakamoto, Shuichi Kaneko, Hideki Ohno, Masao Honda, Masashi Unoura, Seishi Murakami, and Kenichi Kobayashi

First Department of Internal Medicine, Faculty of Medicine (Y.N., S.K., H.O., M.H., M.U., K.K.), and Department of Molecular Biology, Cancer Research Institute (S.M.), Kanazawa University, Kanazawa, Ishikawa, Japan

The hypervariable domain (HVR1) within the N-terminus of the E2 protein of hepatitis C virus (HCV) is known to be variable antigenically during the course of persistent infection. The aim of the study was to detect B-cell epitopes in HVR1 responsible for neutralizing HCV. The B-cell epitopes were analyzed using two series of synthetic peptides: 25 heptapeptides from the most common amino acids within 73 HVR1 sequences, and 216 heptapeptides, the sequences of which cover more than 65% of the 73 HVR1 sequences. Sera from three patients with chronic hepatitis C were tested for reactivity to the synthetic peptide sequences by enzyme-linked immunosorbent assay (ELISA). The post-interferon (IFN) serum of one patient who had a long-term response to treatment reacted specifically with 13 heptapeptides of 216 variable sequences of HVR1. Some of the amino acid sequences (amino acids 398, 399, 400, 404) of the heptapeptides were also found in those deduced from the nucleotide sequences of HCV genomes in the pre-IFN serum. The sera of the other two patients who did not respond to treatment did not react with the 13 heptapeptides. It is concluded that the B-cell epitopes in HVR1 may be relevant for eliminating viremia in the case of the patient who had a good response to treatment. These results suggest that the analysis of the B-cell epitopes recognized in HVR1 may be important in understanding the mechanism of persistent infection and progression of hepatitis.

KEY WORDS: chronic hepatitis C, synthetic peptide, interferon

INTRODUCTION

Hepatitis C virus (HCV) is now recognized as a major agent of chronic hepatitis and liver disease throughout the world [Choo et al., 1989; Kuo et al., 1989; Chien et al., 1992]. At least 50% of infected persons will develop chronic hepatitis [Dienstag, 1983], and 20% of these will go on to develop cirrhosis. The pathogenic mechanism that allows for the persistence of infection and results in a high rate of chronic liver disease is not understood.

In the previous study, the putative envelope (E2) protein of HCV in livers was detected and the possible causal relationship of its expression with progression of hepatitis was demonstrated [Nakamoto et al., 1994]. The hypervariable domain 1 (HVR1) within the N-terminus of the E2 protein is known to contain isolate-specific antibody-binding linear epitopes and to vary antigenically in the course of persistent infection [Weiner et al., 1992; Taniguchi et al., 1993; Kato et al., 1994]. It has been suggested that the variability of the nucleotide sequence of HVR1 may be due to the antibody-mediated immune selection. It has also been suggested that the occurrence of mutations in HVR1 which can escape from the humoral response may be one of mechanisms responsible for HCV persistence. Further, acceleration of immune selection pressure against the HVR1 with the subsequent replication of the variants has also been demonstrated during interferon (IFN) treatment [Weiner et al., 1992]. Studies on patients treated with IFN may therefore be useful for understanding the immune reaction against HVR1.

In this study, B-cell epitopes in HVR1 of three patients treated with IFN were analyzed using 241 synthetic peptides and the corresponding viral amino acid sequence obtained from the patients. The results demonstrated the presence of B-cell epitopes in the HVR1 which may be associated with viral elimination.

MATERIALS AND METHODS

Patients

Three patients (CPH1, female, 52 years old; CPH2, male, 52 years old; CPH3, male, 33 years old) attending

Accepted for publication April 30, 1996.

Address reprint requests to Shuichi Kaneko, M.D., First Department of Internal Medicine, Faculty of Medicine, Kanazawa University, 13-1, Takara-Machi, Kanazawa, Ishikawa 920, Japan.

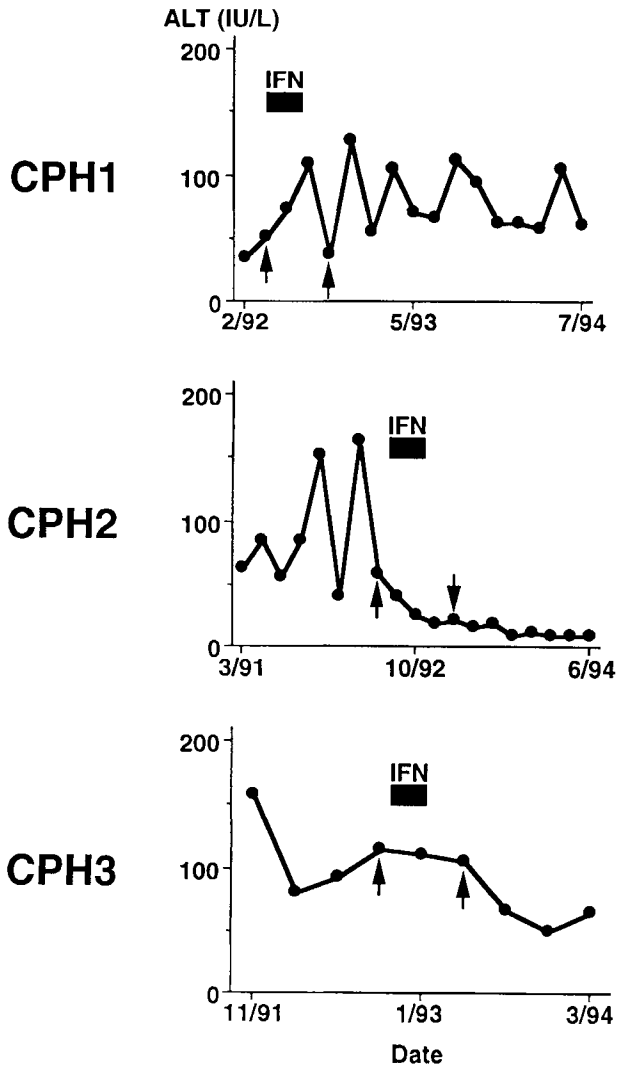

Fig. 1. Course of chronic hepatitis C in three patients treated with recombinant interferon (IFN)-α_{2b}. Arrows show time of blood samplings in this study.

Kanazawa University Hospital were enrolled in this study. Serum samples were obtained from the three patients with chronic persistent hepatitis C before and after treatment with a total dose of 336 MU of recombinant IFN-α_{2b} (Intron-A; Schering-Plough, Japan) (Fig. 1). All of the patients had antibodies against HCV (detected by an HCV second generation assay, Dinabott, Japan), and negative for both hepatitis B surface antigen (HBsAg) and antinuclear antibodies. Serum levels of alanine aminotransferase (ALT) became normal after treatment, and remained normal for over 18 months in patient CPH2. Patients CPH1 and CPH3 did not demonstrate a significant decrease of serum ALT levels during and after the therapy. Serum samples obtained from two patients with chronic hepatitis B were used as a control.

Peptide Synthesis

Alignments of synthetic peptide sequences of HVR1 were made using the solid-phase multi-pin technology [Geysen et al., 1984]. Two sets of heptapeptides deduced from HVR1 sequences of HCV genome registered into GenBank were synthesized. Heptapeptides overlapping by six amino acids, corresponding to CP10 sequence [Okamoto et al., 1992b], were made for a control of the assay of sera obtained from patients with chronic hepatitis C. The same sequences were also synthesized as quality-assurance peptides supplied by the manufacturer (Chiron Mimotopes Pty. Ltd., Australia). All peptides were made in duplicate pins.

ELISA Assay

Enzyme-linked immunosorbent assay (ELISA) of the pin-synthesized peptides was carried out with horse radish peroxidase labeled goat anti-human IgG(Fc) (Gibco BRL, U.S.A.) using diammonium 2,2'-azino-bis [3-ethylbenz-thiazoline-6-sulfonate] (ABTS) as the chromogenic substrate according to the protocols supplied by the manufacturer (Chiron Mimotopes Pty. Ltd.). All sera were diluted 50-fold for the assay. The color produced was read at 405 nm in ELISA reader (ELISA A405 values). All of the sera were tested at least twice and the results were reported as the means of the values, with a standard deviation of less than 5%. A reaction was considered positive if the absorbance given by a peptide was two times higher than the absorbance of background. The background for each serum against each epitopes was calculated as the mean of the lowest 60% of ELISA values given by peptides from the set of the epitope in question. No reaction of horse radish peroxidase labeled goat anti-human IgG(Fc) with all of the peptides tested was confirmed. The quality-assurance pins coupled with reactive and nonreactive peptides and their specific monoclonal antibody were supplied by the manufacturer (Chiron Mimotopes Pty. Ltd.). All quality-assurance peptides and the same sequences synthesized correctly reacted throughout this study.

Isolation and Sequence Analysis of HCV Genome

Using reverse transcriptase-nested polymerase chain reaction (RT-PCR), ten independent HCV clones were isolated from each serum sample also tested in an ELISA assay, and sequenced by the dideoxynucleotide chain termination method, described previously [Kaneko et al., 1989; Nakamoto et al., 1994].

RESULTS

Recognition of the Most Common Amino Acid Sequences by Serum

To know the diversity of HVR1, we compared 73 nucleotide sequences corresponding to amino acid positions 384 to 414 which are registered in GenBank (Table I) [Hijikata et al., 1991; Weiner et al., 1991]. The frequency of the deduced amino acid as indicated in the first column is shown for each position in the sequence. The most common amino acid in each position in HVR1 is listed in the second row.

The antibodies specific for the most common amino acid sequences in HVR1 were detected by using synthetic pep-

TABLE I. The Frequencies of the Amino Acids Deduced From HVR1 Sequences of HCV Genome

Locations of a.a.	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414
Major a.a[a]	E	T	H	V	T	G	G	A	A	A	R	T	T	S	G	L	T	S	L	F	T	P	G	P	S	Q	K	I	Q	L	I
A	5	2	1	3	1	1	9	15	39	30		10	14	9	3	1	23	1			7	3	1	28	8						
C																															
D	2											1									2							1			
E	20						3	1	1		1	1			1													1			
F				1	1				2		2		2	1	1	19			14	62											
G	4		2			67	52	2		30		4		2	35	2		12				1	69					2			
H	4		22				1	4			12	1		3							1	1							3		2
I			3		2	1		2				2				5			5		2			1	1		1	62		1	43
K	3							3			4			1						1					11		34		2		
L			1	4				1				2		4		39		1	48	8		12	2					4	1	69	
M				1	4		2						2	1					3												
N	3		2					3	1			3		5				1		1	4				3	1	27				1
P								1	2						1		1					36	1	34							
Q	7		1		1		2	10	10		10	1		11					2		1	5			5	70	1	1	64		
R	3		16	5			2	3	2	1	36	2		10	9			2			2	3			7		7				
S	4	1	4		16	1	1	11	7	10	5	4	1	18	13		3	56			26	10		9	36				3		
T	12	66	3	10	40			8	1	2	1	39	49	2	9		28			1	28	1			2	2	1				
V	1			47	8	3	1	9	8			3	5		1	6	18					1		1				4		1	27
W																			1											2	
Y			16	1							2			6		1															
X	5	4	2	1																											

[a]The most common amino acids (a.a.) at each location among total 73 isolates registered into GenBank.

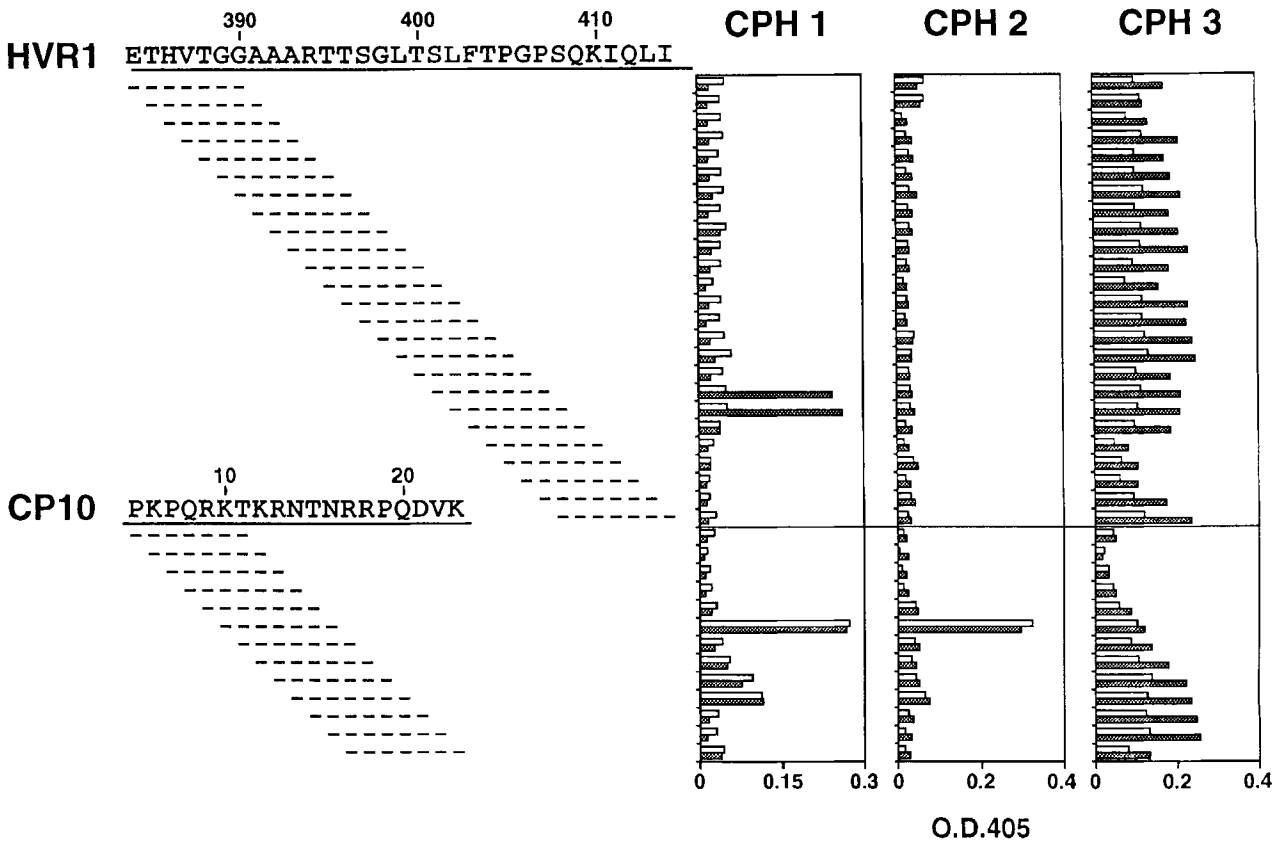

Fig. 2. Recognition of the most frequent amino acid sequences of HCV HVR1 and the sequences of HCV core region (CP10) by pre- and post-interferon (IFN) sera. Numbers show locations of deduced amino acids in the putative structural region of HCV genome. The sequences of the overlapping heptapeptides corresponding to the ELISA A405 values are shown as dots. Open and solid bars show the ELISA values by pre- and post-IFN sera, respectively.

tides. According to the sequences, 25 heptapeptides overlapping by six amino acids were made (Fig. 2), and serum samples from three patients with chronic hepatitis C were analyzed before and after IFN therapy (pre- and post-IFN sera, respectively). Compared with the responses in pre-IFN serum, significantly higher responses to 402-LFTPGP-407 were found in post-IFN serum in patient CPH1 while titers against heptapeptides covering CP10 and other heptapeptides in HVR1 did not change between pre- and post-IFN sera. This finding demonstrated that in this patient antibodies against 402-LFTPGP-407 appeared after IFN therapy while antibodies against other heptapeptides did not change following treatment. No significant response to the peptides in HVR1 was found in either sera from patient CPH2, and high titers against CP10 persisted. Increase of titers against most heptapeptides were seen after IFN treatment in patient CPH3. This elevation corresponded to an increased immunoglobulin level after IFN treatment (1.05 g/dl and 1.26 g/dl in pre- and post-IFN sera, respectively).

Recognition of the Variable Sequences of HVR1

We considered the possibility that no significant signals in responses to the heptapeptides made from the most common amino acids in patients CPH2 and CPH3 might come from the absence of heptapeptide corresponding to authentic antigen in the patients. To make additional heptapeptides which cover as many antigens as possible in a patient's serum, the frequency of each amino acid in each position was calculated. Amino acids 385(T), 389(G), 403(F), 406(G), and 409(Q) were common in more than 80%, and amino acids 387(V), 390(G), 396(T), 401(S), and 402(L) common in more than 65% of the 73 sequences. Antibodies against heptapeptides that contain these highly conserved amino acids were not found in patients CPH2 and CPH3 (Fig. 2).

Amino acids 397, 398, 399, 400, 404, 405, 407, and 408 had greater amount of variation in the 73 sequences. The two variable regions in HVR1 are from amino acid positions 397 to 400, and 404 to 408. If amino acid 397(S), 398(G), 399(L), 400(T), 404(T), 405(P), 407(P), or 408(S) was substituted with one of amino acids {Q, R, A, Y, or N}, {S, R, or T}, {F}, {A or V}, {S or A}, {L or S}, {A}, or {K, A, or R}, respectively, the amino acid sequences from all these combinations would cover more than 65% of the 73 sequences.

Using the strategy of the substitutions listed above, we made 144 and 72 heptapeptides that correspond to

the two variable regions (amino acid postions 396 to 402, and 403 to 409), respectively. As demonstrated Figures 2 and 3, although the serum of patient CPH2 did not show any strong reactions against any of the most common amino acid sequences in HVR1, pre-IFN serum of the patient reacted with two substituted heptapeptides (403-FSLGPKQ-409 and 403-FSLGAKQ-409). Overall titers against the 216 heptapeptides with pre- and post-IFN serum of patient CPH2 were 0.026 ± 0.0068 and 0.032 ± 0.016 (mean ± SD), respectively. Thirteen heptapeptides reacted with post-IFN serum twice as much as with the pre-IFN serum. There were two clusters of sequences which had high titers in post-IFN serum: 1) 396-TXRFTSL-402 and 396-TXTFTSL-402 and 2) 396-TXGLASL-402 and 396-TS(or Q)SLASL-402. There was no significant difference in response to the 216 peptides in sera of patients CPH1 and CPH3 before and after IFN treatments. In two patients with chronic hepatitis B, the serum antibody titers against these peptides were always low.

Sequence Analysis of HCV Genomes

Ten HCV clones were isolated from the pre-IFN serum of patient CPH2 for sequence analysis. Seven of the 10 clones contained the amino acid sequence 396-TGGLASLFSSGSSQ-409 and three clones 396-TGSFVSLFNPGSSQ-409. Although the panel of 216 variable sequences (144 and 72 heptapeptides) screened did not include the heptapeptides 396-TGGLASL-402, 403-FSSGSSQ-409, 396-TGSFVSL-402, or 403-FNPGSSQ-409 derived from the sequences, there were peptides with partial sequence homology at amino acids 398(G), 399(L), 400(A), and 404(S) from major population of HCV clones, or a amino acid 398(S) from minor population (Fig. 3). Sequences of ten HCV clones from the pre-IFN serum of patient CPH3 were also analyzed. Seven different species were identified: three clones with sequence 396-INRLTSFLAPGPSQ-409; two clones with sequence 396-ISRLAGMFSSGASQ-409; a clone with sequence 396-INRLTSFLAPGPPQ-409; a clone with sequence 396-ISRLAGMFSSGAPQ-409; a clone with sequence 396-ISKLTSIFSSGASQ-409; a clone with sequence 396-VSGFTSLFRLGATQ-409; and a clone with sequence 396-ARTIVGMFTSGPSQ-409. We could not obtain the HVR1 fragments from the pre-IFN serum of patient CPH1.

DISCUSSION

The overall mutation rate of the HCV genome has been reported as $\sim 144 \times 10^{-3}$ base substitutions per site per year during an 8.2-year infection in a chimpanzee [Okamoto et al., 1992a]. Sequential amino acid changes in HVR1, however, were observed at a rate of 0.5 to 1.7 amino acids per month in the chronic state of hepatitis [Kato et al., 1992]. HVR1 was known to contain isolate-specific antibody-binding linear epitopes and to vary antigenically during the course of persistent infection [Weiner et al., 1992; Taniguchi et al., 1993]. A recent study demonstrated the existence of two distinct B-cell epitopes (amino acids 394–404 and amino acids 397–407) in HVR1 in a patient with persistent hepatitis C, and emergence of mutations within both epitopes that abrogated recognition by anti-HVR1 antibodies pre-existing in the patient's serum [Kato et al., 1994]. These observations suggest that the occurrence of escape mutations in HVR1, which would be faster than the elimination of the virus by antibodies, may be a mechanism for persistent infection of HCV.

Although IFN is one of the established treatments for chronic hepatitis C, only 25% of IFN-treated patients have long-term beneficial responses to therapy [Tine et al., 1991]. It has been suggested that treatment with IFN could accelerate immune selective pressure against HVR1 of E2 with the subsequent replication of variants [Weiner et al., 1992]. Conversely, patients who have a long-term benefit from IFN may develop neutralizing antibody to HVR1. This could then result in HCV elimination.

In this study, B-cell epitopes in HVR1 of HCV genome were identified using a set of the replacement heptapeptides in an ELISA assay. In the previous study, we speculated that differences in affinity for the native E2 antigen may be due to the diversity of the HVR1 of the antigen [Nakamoto et al., 1994]. Indeed, in patient CPH2, although the sera didn't react with the most frequent amino acid sequences of HVR1, it reacted with 13 heptapeptides when the post-IFN serum was tested with 216 variable sequences. These results suggested that B-cell epitopes recognized in the variable sequences of HVR1 had been overlooked because of the diversity of the sequences. This would account for the apparently low incidence of antibody response to the HCV E2 antigen [Chien et al., 1992].

B-cell epitopes observed in the variable amino acid sequences of HVR1 are suspected of being responsible for neutralizing viremia. Although the post-IFN serum of patient CPH1 strongly reacted with the HVR1 sequence, 402-LFTPGP-407, the patient didn't clinically benefit following therapy. Post-IFN serum of patient CPH2 similarly had strong reactions with 13 heptapeptides. However, in contrast to the outcome of patient CPH1, patient CPH2 had sustained normalization of serum ALT even after 1 year of the cessation of the therapy. If immune reaction is helping in viral elimination from patients whose virus particles were already decreased by IFN treatment [Shindo et al., 1991; Reichard et al., 1994; Davis et al., 1994], there are at least three explanations for the difference of outcome in these two patients. Although the antibodies against 402-LFTPGP-407 in patient CPH1 were responsible for elimination of the viruses containing the identical sequence [Taniguchi et al., 1993; Kato et al., 1994], rate of mutation in the region may have been faster than the production of the antibodies. Also, a pre-existing variant virus that was not recognized by the humoral immune attack may have been selected and have overgrown despite apparent antibody response in patient CPH1. Another explanation is that antibodies against 402-LFTPGP-407 were not important for the elimination. However, in patient CPH2, the antibodies against the 13 heptapep-

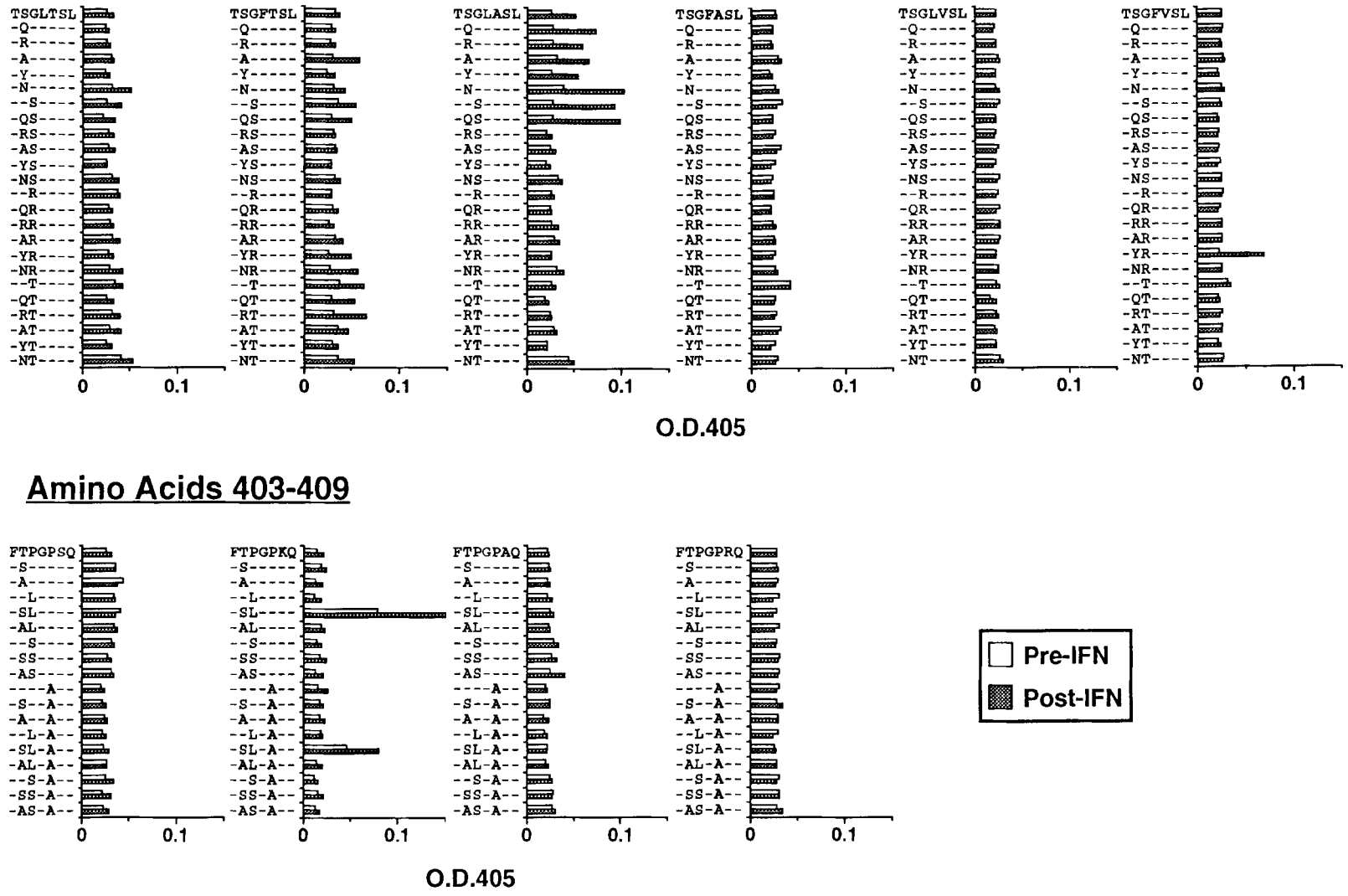

Fig. 3. Recognition of the variable sequences of HVR1 (amino acids 396–402 and amino acids 403–409) by pre- and post-IFN sera of patient CPH2. The most frequent sequences of HVR1, 396-TSGLTSL-402 and 403-FTPGPSQ-409, were substituted as upper and lower rows, respectively. The amino acid sequences were compared to the sequences shown on the top line. Identical amino acids are shown as dots. Open and solid bars show the ELISA values by the pre- and post-IFN sera, respectively.

tides may have been important in viral elimination. In addition, by inducing the production of these antibodies, IFN may have been beneficial for the patient. Finally, these antibodies might be unrelated to viral elimination. Certainly, it is difficult to demonstrate the role of overall B-cell epitopes in HVR1 in viral elimination due to the variation in the clinical and virological status of the patients.

We demonstrated that two regions in HVR1 that may be responsible for the humoral elimination of HCV. Especially, HCV sequences which contain amino acids 398-GLA-400 were found to be present in the serum before treatment, and to be a highly reactive peptide with post-IFN serum. Further studies using large number of cases will be necessary to define the role of antibodies against the region on viral elimination. Although HCV infection is known to become chronic, some of the patients infected with HCV spontaneously recover from the infection. Even after becoming chronic carriers, others can clear the infection following treatment with IFN. Analysis of B-cell epitopes recognized in HVR1 in E2 of HCV will be important for understanding of the neutralization of the viremia and possibly developing a HVR1 vaccine.

ACKNOWLEDGMENTS

We thank Dr. Michael E. Brown and Dr. Kyong-Mi Chang for discussion and careful reading of the manuscript.

REFERENCES

Chien DY, Choo Q-L, Tabrizi A, Kuo C, McFarland J, Berger K, Lee C, Shuster JR, Nguyen T, Moyer DL, Tong J, Furuta S, Omata M, Tegtmeier G, Alter H, Schiff E, Jeffers L, Houghton M, Kuo G (1992): Diagnosis of hepatitis C virus (HCV) infection using an immunodominant chimeric polyprotein to capture circulating antibodies: Reevaluation of the role of HCV in liver disease. Proceedings of the National Academy of Sciences of the United States of America 89:10011–10015.

Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.

Davis GL, Lau JY-N, Urdea MS, Neuwald PD, Wilber JC, Lindsay K, Perrillo RP, Albrecht J (1994): Quantitative detection of hepatitis C virus RNA with a solid-phase signal amplification method: Definition of optimal conditions for specimen collection and clinical application in interferon-treated patients. Hepatology 19:1337–1341.

Dienstag JL (1983): Non-A, non-B hepatitis: Recognition, epidemiology and clinical features. Gastroenterology 85:439.

Geysen HM, Meloen RH, Barteling SJ (1984): Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proceedings of the National Academy of Sciences of The United States of America 81:3998–4002.

Hijikata M, Kato N, Ootsuyama Y, Nakagawa M, Ohkoshi S, Shimotohno K (1991): Hypervariable regions in the putative glycoprotein of hepatitis C virus. Biochemical and Biophysical Research Communications 175:220–228.

Kaneko S, Miller RH, Feinstone SM, Unoura M, Kobayashi K, Hattori N, Purcell RH (1989): Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. Proceedings of the National Academy of Sciences of The United States of America 86:312–316.

Kato N, Ootsuyama Y, Ohkoshi S, Nakazawa T, Sekiya H, Hijikata M, Shimotohno K (1992): Characterization of hypervariable regions in the putative envelope protein of hepatitis C virus. Biochemical and Biophysical Research Communications 189:119–127.

Kato N, Ootsuyama Y, Sekiya H, Ohkoshi S, Nakazawa T, Hijikata M, Shimotohno K (1994): Genetic drift in hypervariable region 1 of the viral genome in persistent hepatitis C virus infection. Journal of Virology 68:4776–4784.

Kuo G, Choo Q-L, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo M, Lee W-S, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW, Houghton M (1989): An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364.

Nakamoto Y, Kaneko S, Honda M, Unoura M, Cheong J, Harada A, Matsushima K, Kobayashi K, Murakami S (1994): Detection of the putative E2 protein of hepatitis C virus in human liver. Journal of Medical Virology 42:374–379.

Okamoto H, Kojima M, Okada S-I, Yoshizawa H, Iizuka H, Tanaka T, Muchmore EE, Peterson DA, Ito Y, Mishiro S (1992a): Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: Variability and stability. Virology 190:894–899.

Okamoto H, Tsuda F, Machida A, Munekata E, Akahane Y, Sugai Y, Mashiko K, Mitsui T, Tanaka T, Miyakawa Y, Mayumi M (1992b): Antibodies against synthetic oligopeptides deduced from the putative core gene for the diagnosis of hepatitis C virus infection. Hepatology 15:180–186.

Reichard O, Foberg U, Fryden A, Mattsson L, Norkrans G, Sonnerborg A, Wejstal R, Yun Z-B, Weiland O (1994): High sustained response rate and clearance of viremia in chronic hepatitis C after treatment with interferon-a2b for 60 weeks. Hepatology 19:280–285.

Shindo M, Di Bisceglie A, Cheung L, Wai-Kuo Shih J, Cristiano K, Feinstone S, Hoofnagle J (1991): Decrease in serum hepatitis C viral RNA during alpha-interferon therapy for chronic hepatitis C. Annals of Internal Medicine 115:700–704.

Taniguchi S, Okamoto H, Sakamoto M, Kojima M, Tsuda F, Tanaka T, Munekata E, Muchmore EE, Peterson DA, Mishiro S (1993): A structurally flexible and antigenically variable N-terminal domain of the hepatitis C virus E2/NS1 protein: Implication for an escape from antibody. Virology 195:297–301.

Tine F, Magrin S, Craxi A, Pagliaro L (1991): Interferon for non-A, non-B chronic hepatitis. A meta-analysis of randomised clinical trials. Journal of Hepatology 13:192–199.

Weiner AJ, Brauer MJ, Rosenblatt J, Richman KH, Tung J, Crawford K, Bonino F, Saracco G, Choo Q-L, Houghton M, Han JH (1991): Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. Virology 180:842–848.

Weiner AJ, Geysen HM, Christopherson C, Hall JE, Mason TJ, Saracco G, Bonino F, Crawford K, Marion CD, Crawford KA, Brunetto M, Barr PJ, Miyamura T, McHutchinson J, Houghton M (1992): Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: Potential role in chronic HCV infections. Proceedings of the National Academy of Sciences of The United States of America. 89:3468–3472.